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Genetic instability is a hallmark of tumor development. Mechanisms for maintenance of genomic stability are heterogeneous and identification of the genes responsible a critical goal of cancer biologists. The very large number of genetic alteration in breast tumors and genetic heterogeneity, even within a single breast tumor, strongly suggests that some mutator mechanism must be involved in breast tumorigenesis. Our hypothesis is that a mutator mechanism contributes to the development of breast cancer. However, since breast tumors do not display an obvious phenotype (such as microsatellite instability) that signals the presence of a mutator defect, another scheme to identify defects in repair genes and their targets is necessary. Thus, our first objective is to use a novel yeast model system to identify genes that are previously unrecognized targets of mutator mechanisms and to determine whether these genes are altered in breast tumors. Our second objective is to identify genes that function as novel mutators in the yeast system then evaluate whether any are altered in breast tumors. If we are fortunate enough to identify mutator genes and their targets involved in breast tumorigenesis, it will revolutionize breast cancer genetics.

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Introduction:

All cells are subject to continual DNA damage. For this reason, elaborate pathways have been developed to monitor DNA damage and to coordinate cell cycle progression with DNA repair. To date, over 70 genes involved in DNA damage surveillance and repair have been identified (Wood, Mitchell et al. 2001). These genes include those involved in mismatch repair, homologous recombination, non-homologous end joining, and signaling cascades that respond to DNA damage. However, only a few of these genes have been shown to be associated with breast tumor development. The very large number of genetic alterations in breast tumors, and genetic heterogeneity even within a single breast tumor, strongly suggest that other repair genes must play a role in breast tumorigenesis.

Our hypothesis is that a mutator mechanism contributes to the development of breast cancer. However, since breast tumors do not display a phenotype (such as microsatellite instability) to signal the presence of repair defects, another scheme to identify such genes and their targets is necessary. Our rationale for this project is based on the evolutionary conservation of DNA repair systems between yeast and humans. This conservation enabled us to detect and measure, in yeast strains, the increase in mutation rates in human tumor suppressor genes due to defective mismatch repair.

The objective of our research is to use a novel yeast-based screen to identify genes that are previously unrecognized targets of mutator mechanisms and genes that function as novel mutators. Our aims include generating a high quality, complex breast cDNA library, using this library in a novel, yeast-based screen designed to identify genes in the breast transcriptome that are targets of mutators, and screening for novel mutator genes using known tumor suppressor genes as targets.

The scope of our research includes the evaluation of newly identified genes to determine whether any are altered in breast tumors. Discovery and functional assessment of these genes is essential for understanding the biology of breast cancer and for clinical applications, including identification of therapeutic targets, early breast cancer detection and improved prediction of breast cancer risk and disease course.

Body:

Statement of Work for DAMD17-02-1-0615 as actually funded.

Technical objective 1: ***Construction of a high quality breast cDNA library***

Months 1-6: Culture normal breast mammary epithelial cells from dissected breast reduction material from premenopausal women. Confirm quality of cultures using immunohistochemical methods. Isolate total RNA, generate poly A+ RNA and convert to cDNA using standard techniques. Clone material into lambda TripLEX. Transduce lambda TripLEX pahgemid library into *E. coli* BNN123 and isolate plasmid DNA.

Studies and Results, June 01, 2002-June 30, 2003

We proposed to construct a high quality normal breast cDNA library using reduction material from 3-4 premenopausal women. Normal mammary epithelial cells have been cultured, the quality of the cultures has been confirmed, and poly A+ RNA has been isolated.

Our rationale for pooling cDNA from different premenopausal women was to normalize the library for genetic contributions that are unique to a particular individual. However, in reviewing our rationale for constructing the breast library solely with cDNA from premenopausal women, we realized that the library should be 'complex' meaning that it should also consist of cDNA from normal epithelial cells from premenopausal women (as proposed), and from postmenopausal women and breast tumors. Construction of such a complex breast cDNA library allows us to perform a more complete screen.

We have cultured and isolated poly A+ RNA from a number of breast tumor cell lines including those positive and negative for the estrogen receptor. In addition, we have isolated poly A+ RNA from breast tumor cell lines that have no wild-type BRCA1 expression, cell lines that have reduced BRCA1 expression, and cell lines that have wild-

type BRCA1 expression. Thus, genes that are targets of mutator mechanisms in premenopausal, postmenopausal, and breast tumor cells will be identified.

Technical objectve 2: ***In vivo construction of a breast cDNA library in the yeast vector pCI-HA***

Months 7-8: PCR amplify the normal breast cDNA library with PCR primers to facilitate gap repair. Linearize pCI-HA. Use high efficiency yeast transformation protocol to transform yeast strain *msh2* and *mlh1* with linearized plasmid and PCR amplified cDNAs.

Studies and Results, June 01, 2002-June 30, 2003

We originally proposed to construct the breast cDNA library during months 7-8. However, this objective was delayed approximately 4 months to allow for the culture of additional breast cDNAs to make the complex breast cDNA library (see Technical objective 1, Studies and Results). The library is presently under construction.

Technical objective 3: ***Screen for targets of mutator mechanisms in the breast transcriptome.***

Months 9-18: Perform dual plating of the normal breast cDNA library in *msh2* on -Leu/-Ura and -Leu/FOA plates to constrain mutator phenotype. Replica clones onto –Leu plates to allow for muation of target sequences. Select for clones that have been disrupted due to defective mismatch repair by replica plating onto either –Leu/FOA or –Leu/-Ura. Repeat the screen in the *mlh1* strain.

Studies and Results, June 01, 2002-June 30, 2003

We have obtained yeast strains defective for *msh2* and *mlh1*. We have confirmed that these strains display a mutator phenotype by transforming them with control plasmids (those containing known targets of these mutator genes) and selecting for mutation events by plating transformants on media containing –Leu/FOA. Mutation rates have been determined and agree with published results. Having confirmed the phenotype of the yeast strains, we are ready to begin the screen to identify targets of these mutators. Because this objective covers months 9-18, we will include progress on this objective in our Annual Report for year 2 of this grant.

Technical objective 4: ***Identification of target genes and mutations and confirmation of hypermutability in candidate genes***

Months 19-20: Rescue cDNA clones that are disrupted in *msh2* or *mlh1* strains using standard yeast plasmid rescue protocols. Sequence cDNA inserts to identify gene and mutation. Use candidate cDNA clones to retransform naïve *msh2* and *mlh1* strains and determine mutation rate using date from several fluctuation analyses.

Studies and Results, June 01, 2002-June 30, 2003

A summary of studies and results for this objective (which covers months 19-20) will be included in the Annual Report for year two of this award.

Technical objective 5: ***Analysis of candidate genes in sporadic breast tumors***

Months 21-24: Prepare microdissected material from sporadic and inherited breast tumor samples. Isolate DNA from microdissected tumor and normal tissues (normal breast tissue or blood). Sequence candidate genes in tumor and normal DNAs to Identify mutations. Repeat procedure for sporadic ovarian tumor samples.

Studies and Results, June 01, 2002-June 30, 2003

A summary of studies and results for this objective (which covers months 21-24) will be included in the Annual Report for year two of this award.

Technical objective 6: ***Screen for novel mutator genes***

Months 25-32: From the haploid set of SGP deletion strains, remove strains deleted for known mutator genes. Pool remaining clones in subsets. Use high efficiency yeast transformation protocols to introduce plasmid pHJ3. Repeat protocol for plasmids pHJ4 and pHJ9. Identify strains with a mutator phenotype by plating onto –Ura/-Leu to constrain mutator phenotype. Replica plate clones onto –Leu to allow for MSI-related mutations to occur in target sequences. Select for strains with a mutator phenotype by replica plating onto –Leu/FOA plates. Identify deleted gene in clones, which display a mutator phenotype by PCR amplification and sequencing. Determine mutation rates as described.

Studies and Results, June 01, 2002-June 30, 2003

A summary of studies and results for this objective (which covers months 25-32) will be included in the Annual Report for year three of this award.

Technical objective 7: ***Analysis of candidate mutator genes in sporadic breast tumors.***

Months 33-36: Use bioinformatic analyses to identify human homologs of novel yeast mutator genes. Prepare microdissected material from sporadic and inherited breast tumor samples. Isolate DNA from microdissected tumor and normal tissues (normal breast tissue or blood). Sequence candidate genes in tumor and normal DNAs to Identify mutations. Repeat procedure for sporadic ovarian tumor samples.

Studies and Results, June 01, 2002-June 30, 2003

A summary of studies and results for this objective (which covers months 33-36) will be included in the Annual Report for year three of this award.

Key Research Accomplishments

- We have cultured and confirmed the quality of poly A+ RNA from cultured normal mammary epithelial cells from pre- and postmenopausal women. In addition, we have cultured and confirmed the quality of poly A+ RNA from a variety of breast tumor cells lines

- We have constructed a high quality complex breast cDNA library using poly A+ RNA isolated from normal mammary epithelial cells from pre- and postmenopausal women and from various breast tumor cell lines.

- We have confirmed the mutator phenotype of yeast strains defective in *msh2* and *mlh1*.

- We have continued to expand our breast tissue bank. To date, our bank consists of over 350 matched breast tumor and normal tissue samples.

Reportable Outcomes

We report here the progress of the first year of a three-year award. Thus, to date, we have no reportable outcomes

Conclusions

Tumor development is the result of an imbalance between mechanisms controlling gene regulation and genomic stability. Genomic stability is under genetic control. Thus, identification of genes that maintain stability is a goal of cancer biologists. Because a mutator mechanism contributes to the development of breast cancer, we have initiated research designed to identify heretofore, unrecognized targets of mutator mechanisms as well as novel mutators and

to determine whether these genes are altered in breast tumors. If we discover novel targets of mutators and/or novel mutators with consequences for breast tumor development, we will open new pathways for investigation into detection and treatment of breast cancer.

References:

Wood, R. D., M. Mitchell, et al. (2001). "Human DNA repair genes." Science **291**(5507): 1284-9.